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Placental alkaline phosphatase in developing normal and abnormal gonads and in germ-cell tumours

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Summary. The evolution of the gonads during intra-uterine development has been followed by immunohistochemical demonstration of placental-like alkaline phosphatase (PLAP) at the germ-cell level. PLAP immunopositivity was restricted to the limited period when germ cells were not surrounded by granulosa or Sertoli cells. Abnormal fetuses or neonates presenting with chromosomal anomalies frequently had disorganized gonads where germ cells retained their membrane PLAP immunopositivity. This abnormal immunopositivity is similar to that expressed by abnormal germ cells in testicular in situ carcinoma, in gonadoblastoma (case of 45,XO/46,XY mosaic) and in seminoma. The pattern of positivity for other germ-cell tumours was highly variable. We suggest that in abnormal gonads, dysgenetic or neoplastic, an early embryonic property is retained by abnormal germ cells. Its importance in the process of neoplastic induction remains to be defined.

Key words: Placental alkaline phosphatase – Germ cells – Embryo – Chromosome anomalies – Germcell tumours

Introduction

Placental-like alkaline phosphatase (PLAP) immunoreactivity has been demonstrated recently in various testicular tumours, particularly in seminomas, where the percentage of immunohistochemically positive cases exceeds 90% (Paiva et al. 1983; Mostofi et al. 1987; Wick et al. 1987).

In a retrospective study, we noted that testicular in situ carcinomas (CIS) was present surrounding germ-cell tumours, in a very large number of cases. PLAP immunopositivity was demonstrated at the cell membrane of almost all intratubular atypical germ cells (Hustin et al. 1987). This finding was further substantiated by the study of several cases of isolated testicular CIS which also showed with PLAP immunopositivity, in the absence of any invasive germ-cell tumour. This led us to consider that intratubular neoplastic germ cells displayed a "new" biochemical property, not previously recognized at the level of normal spermatogonia.

In a preliminary study, we demonstrated that primitive germ cells (those identified in the genital ridge of the growing embryo) were also PLAP-positive (Hustin et al. 1987). We thus considered that these findings pointed towards re-expression by neoplastic cells of a property of true precursor embryonic cells. The purpose of the present study is further to characterize PLAP membrane positivity on embryonic germ cells and to follow its evolution at different ages of intra-uterine life and in the neonatal and infantile period. It is well known that gonadal organization during embryonic life is under the influence of sex chromosomes and possibly also of some autosomal control (Kennedy et al. 1977; Cattanach 1987). As dysgenetic gonads frequently undergo malignant transformation during adult life (Scully 1981; Muller et al. 1985), we thought it valuable to trace the fate of germ cells in such abnormal organs. We also studied a number of germ-cell tumours.

Materials and methods

Tissue blocks from 132 cases were selected from our files. They were divided into four groups:

1. Normal gonads (testes or ovaries) obtained from 34 embryos, fetuses and neonates either as products of voluntary interruptions of pregnancy or as parts of fetal and neonatal autopsies. Ten testicular biopsies from normal adults with male infertility were also included.
2. The second group comprised 17 cases of genetic disorders: 4 were anomalies of sex chromosomes, 2 cases were triploid (both 69,XXY) and there were 11 autosomal trisomies (7 trisomy 18 and 4 trisomy 13). All these were neonatal autopsy cases except for one gonadal biopsy from a 45,XO/46,XY individual, aged 17, presenting with a gonadoblastoma.
3. Testicular biopsies obtained at the time of late surgery from 14 cryptorchid boys represented the third group.

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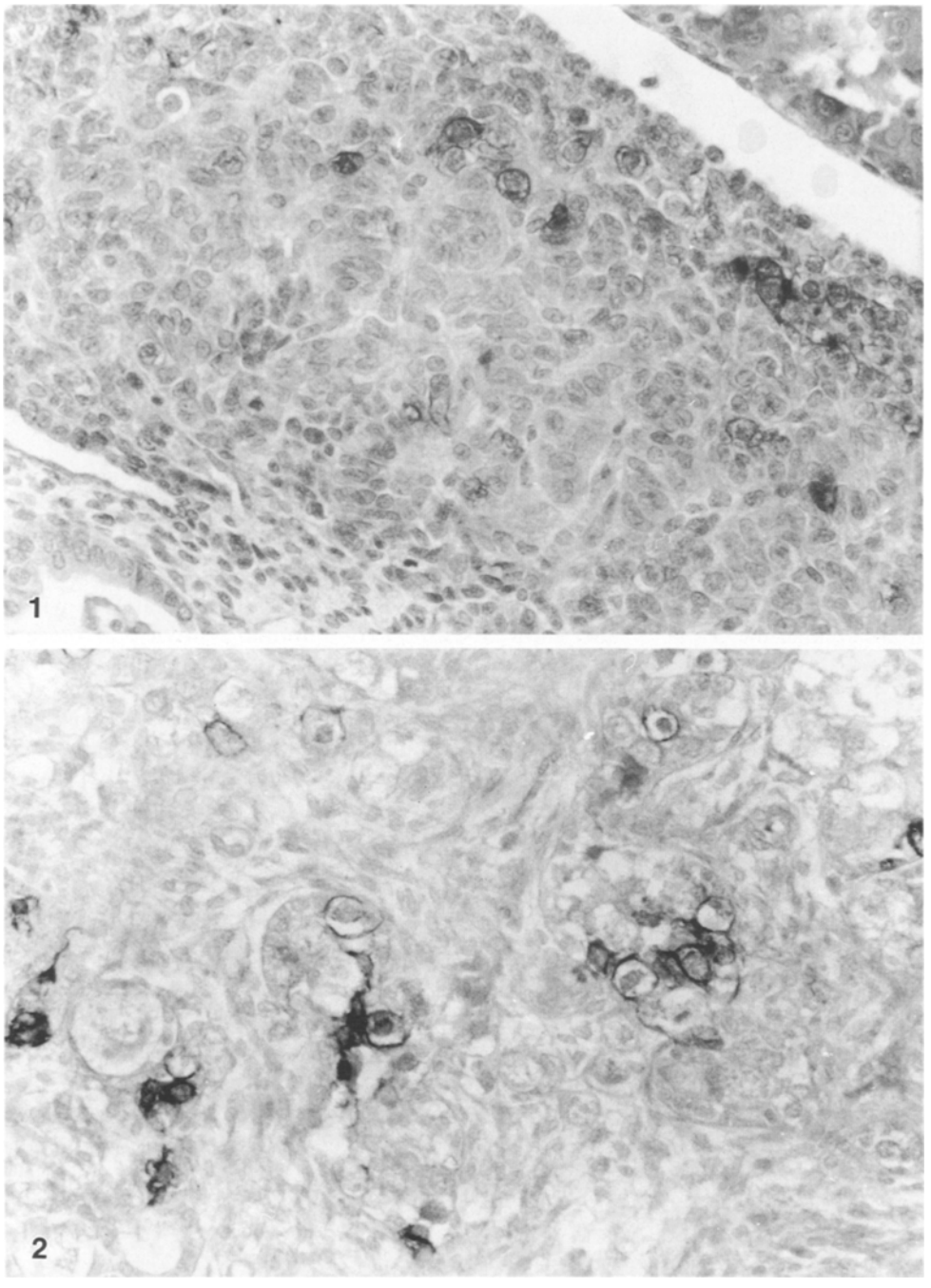


Fig. 1. First trimester embryo, primitive gonad. Numerous germ cells are PLAP immunostained in the cortex PLAP polyclonal immunostaining and Harris haematoxylin counterstain, ×125

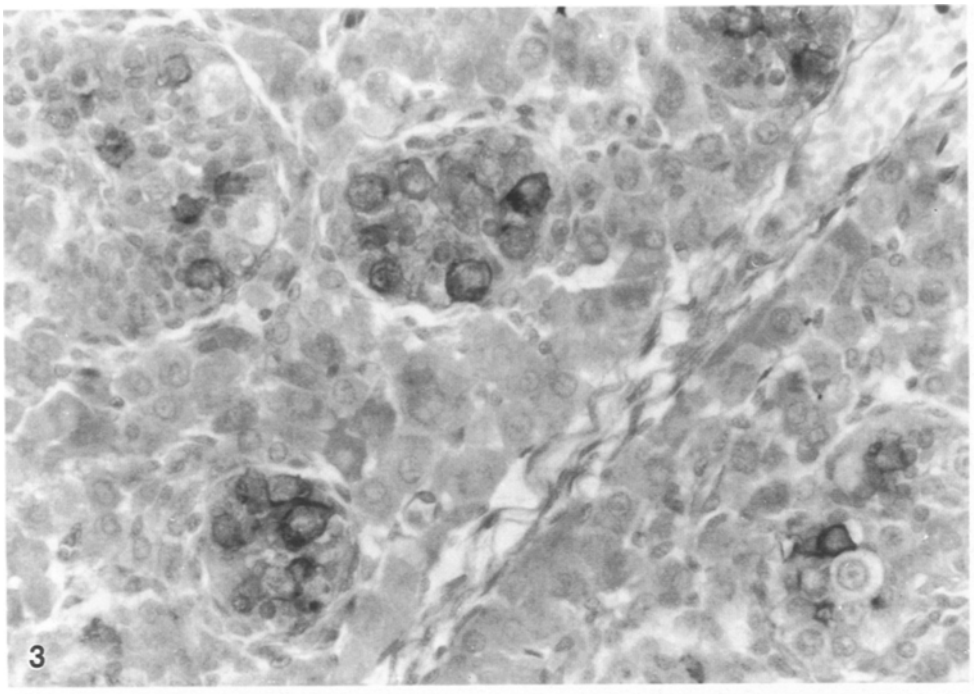
Fig. 2. Ovary from trisomy 18 stillborn. Interspersed with normal negative ovocytes, are several groups of free PLAP-positive germ cells. PLAP polyclonal immunostaining and Harris haematoxylin counterstain, ×250

4. The fourth series consisted of germ cell tumours of the testis.

In 5 cases, the presence of intratubular atypical germ cells (C1S) was the sole finding. In 50 specimens, invasive germ-cell tumours were present. There were 26 seminomas, with 1 so-called spermatocytic seminoma, 18 embryonal carcinomas, 2 choriocarcinomas and 4 immature teratomas. Finally, 2 cases of malignant lymphoma invading the testis were examined.

A polyclonal antiserum (raised in rabbits) directed against PLAP (Dako-Prosan, Ghent, Belgium) was used at a 1/1000 dilution. Two monoclonal antibodies were also used: H17E2 (Unipath, Bedford, UK) a purified mouse antibody with an initial concentration of 0.2 mg/ml IgG_1 (Travers and Bodmer 1984), was used at a 1/1000 dilution; a M1G-P1 (Innogenetics, Antwerp, Belgium), was obtained as a culture supernatant containing the antibody of the IgG_1 kappa class, and used at a 1/50 dilution in 0.05 M phosphate-buffered saline (PBS) containing 1% bovine serum albumin (Nouwen et al. 1987).

All necropsy specimens had been fixed in buffered formalin, while testicular biopsies and germ-cell tumours were fixed in Bouin's fluid. The specimens were further embedded in Paraplast and semi-serially sectioned at 5 μm. In preliminary investigations, we had noticed that PLAP monoclonal antibodies did not react on such specimens. Hence, we also used tissue slices which had been snap-frozen in liquid nitrogen and further sectioned at −20° C in a cryostat. These frozen sections were immunostained with the monoclonal antibodies. The frozen sections were obtained from germ-cell tumours: 1 isolated CIS, 3 seminomas and 1 embryonal carcinoma.



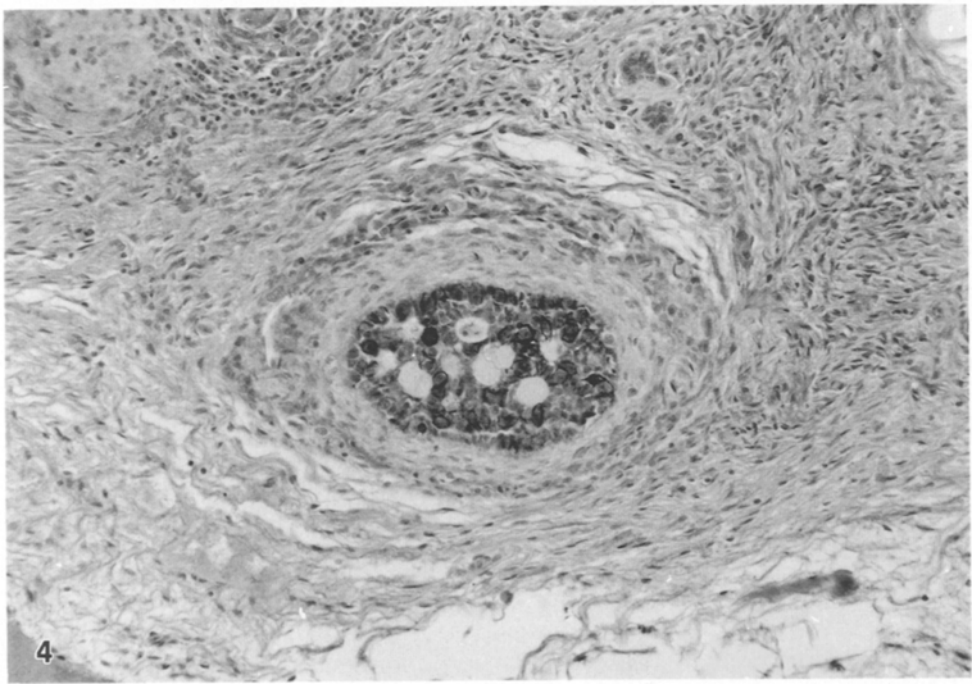


Fig. 3. Fetal testis, triploid syndrome. Spermatogonia within tubules are PLAP-positive. PLAP polyclonal immunostaining and Harris haematoxylin counterstain, ×250

Fig. 4. 45,XO/46XY individual with gonadoblastoma nest in the ambiguous gonad. Note the numerous PLAP-positive cells (tumour germ cells?). PLAP polyclonal immunostaining and Harris haematoxylin counterstain, ×125

The immunocytochemical technique (Taylor 1986) included overnight incubation of the slides with the appropriate dilution of the selected antibody at 4° C. After thorough washing with PBS, the second species-specific antibody coupled with biotin (swine anti-rabbit for polyclonals, goat anti-mouse for monoclonals; Dako-Prosan, Ghent, Belgium) was layered on slides at a 1/100 dilution for 30 min.

After washing with buffer, the ABC complex (Vectastain, Vector, Burlingame, Calif., USA) was added. It consisted of a complex of avidin with biotin complexed with horseradish peroxidase. Detection of the immunopositive sites was accomplished with perhydrol and 3-3′ diaminobenzidine followed by counterstaining with Harris haematoxylin. Controls consisted of step slides incubated with non-immune rabbit serum replacing the primary antiserum.

Results

In gonads from first-trimester embryos, large cells with vesicular nuclei, could be identified within the genital ridge, long before sex-cord proliferation took place. These cells, as a rule, were characterized by a membrane rim of PLAP positivity (Fig. 1). The evolution was some what different in chronology in male and female gonads. The first sex-cord proliferation occurred earlier in testes than in ovaries: male germ cells were entrapped earlier, and began to lose their PLAP immunopositivity during the first half of the second trimester. By contrast, oogon-

ia remained free and PLAP positive until the secondary sex-cord maturation. Afterwards the immunocytochemical reaction became progressively negative.

The gonads from the trisomic (13 and 18) fetuses and neonates were ovaries. It was clear that the second sex-cord proliferation was disorganized and largely incomplete. Therefore, negative oogonia surrounded by a single layer of future granulosa cells co-existed with free positive germ cells in a highly cellular stroma (Fig. 2). The ratio of primitive germ cells to mature oogonia varied widely from one case to another.

Both triploid cases were males (69,XXY). Testicular tubular maturation was normal but spermatogonia within the tubules displayed strong PLAP immunopositivity (Fig. 3).

The gonads of 45,XO individuals still possessed recognizable germ cells at term but they were considerably less numerous and many of them appeared pre-necrotic. PLAP immunostaining was present but irregular and much weaker than in trisomic cases. The last chromosomal anomalies were 45,XO/46,XY mosaics. Their gonads were usually reduced in size. Scattered germ cells were PLAP positive among aggregates of negative primitive Sertoli cells.

The incidence of PLAP immunopositivity in the different specimens of chromosomal anomalies is shown in Table 1.

As a rule, testes and ovaries of normal neonates and infants were totally devoid of any immunopositivity for PLAP as were testicular biopsies from prepubertal boys and normal adult men. Biopsies obtained from cryptorchid patients undergoing late operation were also negative though the spermatogonia were usually larger than in normal descended testes.

The gonadoblastoma case occurring in a 16-year-old phenotypically female individual with 45,XO/46,XY karyotype was characterized by many large cells with membrane PLAP positivity enmeshed in groups of negative dark cells (granulosa cells?) (Fig. 4). It was interesting to note a marked inflammatory reaction with granuloma formation in this case progressively destroying the tumour nests. PLAP positive cells did not seem to be attacked. Lastly, in the series of germ-cell tumours, it was demonstrated that PLAP immunopositivity was present in the cells of almost every case of CIS presenting either as an isolated finding or accompanying true invasive germ-cell tumours (Table 2). Interestingly enough, in some cases, CIS was clearly PLAP positive while the non-seminomatous germ-cell tumour in the vicinity was negative. As regards the latter, the percentage of immunopositivity is summarized in Table 3. It appears that seminomas are associated with a 96% incidence of PLAP positivity (Fig. 5). In those cases where tumour tissue had been frozen, the same immunocytochemical pattern could be demonstrated either with the polyclonal or with the two monoclonal antibodies. Embryonal carcinomas were also frequently PLAP-positive (2/3 of cases); however, only a limited number of tumour cells (in some cases less than 5%) were immunostained (Fig. 6). Both choriocarcinoma cases were PLAP-positive only in syncytiotrophoblastic areas.

Table 1. Genetic anomalies: presence of isolated PLAP-positive germ cells

I.	Autosomal trisomies	9/11
II.	Triploid syndromes	2/2
III.	Disorders of sexual chromosomes	[a]

[a] 45,XY: 0/1
45,XO/46,XY ($n=2$): germ cells are involuting a faint rim of positivity is barely discernible
Feminizing testis with gonadoblastoma: 1/1

Table 2. Carcinoma in situ PLAP-like-immunopositivity (intra-tubular atypical germ cells)

Accompanying obvious germ-cell tumour	21/22[a]
Isolated finding	5/5

[a] Very weak staining in three cases, all others being rated + + to + + +

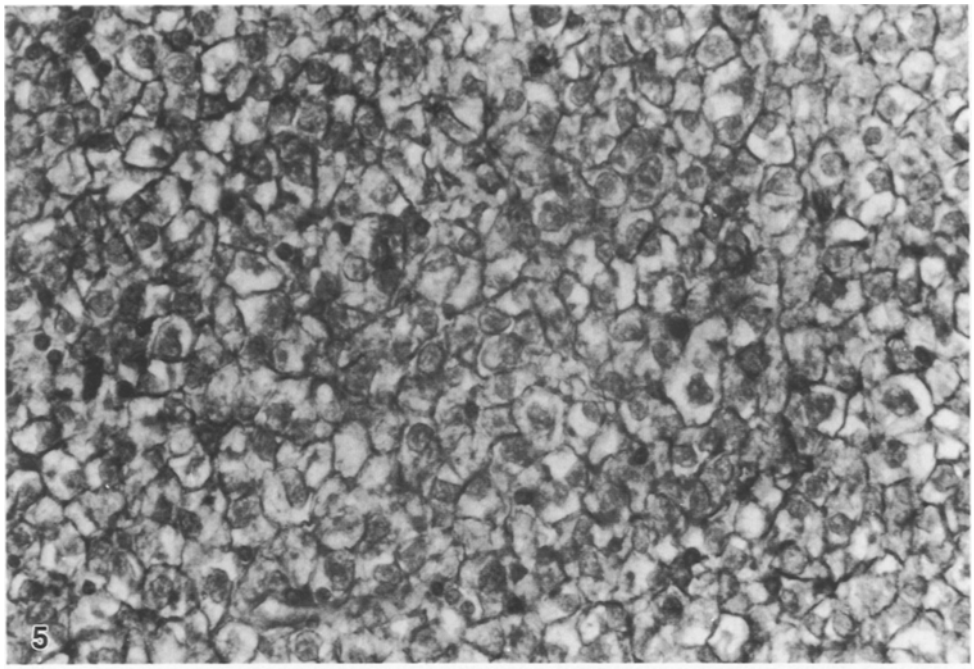
Table 3. Germ-cell tumours, PLAP-like immunoreactivity

	No. of Positive Cases	%
Seminomas	24/25	96
Spermatocytic seminoma	0/1	0
Embryonal carcinomas	11/18	61
Choriocarcinomas	2/2	100
Immature teratomas	0/4	0
Lymphomas	0/2	0

Discussion

In 1953, Mc Kay et al. demonstrated that primitive germ cells possessed significant alkaline phosphatase activity. By a simple histochemical stain, these authors could follow the migration of germ cells along the genital ridge and their clustering in the future gonads. It was only very recently that immunohistochemistry confirmed this pioneering work. We demonstrated (Hustin et al. 1987) that embryonic germ cells, either still migrating or already within the primitive gonads, reacted at the cell membrane with polyclonal antiserum directed against placental alkaline phosphatase. This finding permitted us to suggest that "free" germ cells must be under strict proliferation control and that PLAP could be implicated in this regulation in a manner similar to that postulated for second- and third-trimester placenta (Risk and Johnson 1985).

Gonadal development and differentiation depends on several factors: formation of a testis implies that a Y chromosome is present and that it contains a complete testis differentiation gene (Tdg) (Vergnaud et al. 1986). In the absence of a Y chromosome, differentiation towards ovary occurs (Cattanach 1987). Further development is obviously controlled at least partly by autosomes: several studies (Kennedy et al. 1977; Moerman



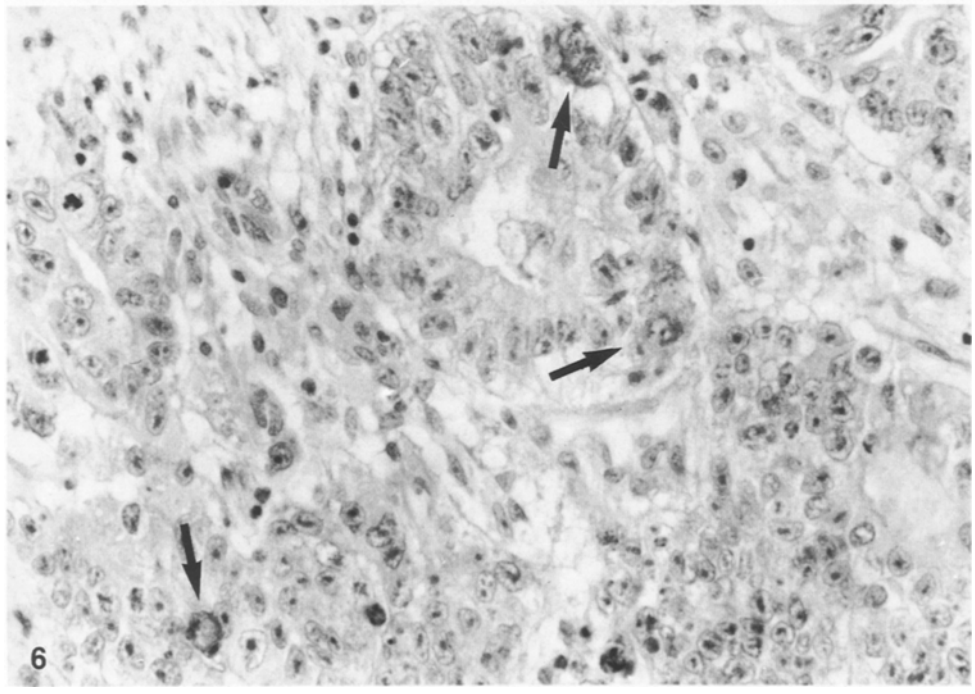


Fig. 5. Seminoma. All tumour cells are PLAP-positive at the cell membrane. PLAP polyclonal immunostaining and Harris haematoxylin counterstain, ×250

Fig. 6. Embryonal carcinoma of the testis. Some isolated cells (*arrows*) are PLAP-positive. PLAP polyclonal immunostaining and Harris haematoxylin counterstain, ×250

et al. 1988) have clearly demonstrated that neonates presenting with trisomies 13 and 18 are predominantly females and that their ovaries are almost always abnormal with persistence of isolated "free" germ cells in the vicinity of granulosa cords and of a limited number of normal oocytes.

In such cases, our present study demonstrated persistent PLAP immunopositivity at the cell membrane of "free" germ cells this contrasting with the total negativity of oocytes. In other fetuses, presenting with various anomalies of sex chromosomes, we also showed PLAP immunopositivity of "free" germ cells. In 45,XO individuals, germ cells eventually degenerated but, at term, they still could be identified with a faint rim of PLAP immunopositivity. If Tdg is expressed partly (in 45,XO/46,XY mosaics), gonadal development was only partly impaired, but free germ cells could be identified in the stroma; they expressed membrane PLAP.

The immunohistochemical similarity between ovaries from fetuses displaying trisomy 13 or 18 and the gonads affected by disorders of sex chromosomes is striking. Intersex gonads have a well-known tendency to develop

germ-cell tumours (Scully 1981; Muller et al. 1985). Our gonadoblastoma case is particularly interesting in this respect: this neoplasm develops in a dysgenetic gonad, as a precursor of true malignant germ-cell tumours, and displays PLAP immunopositivity within the germ-cell component. We suggest that persistence of PLAP positive germ cells at a later stage of gestation or even after birth reflects a disorder of sex-cord formation. It can be postulated that this could be a key factor for later tumour growth. It is conceivable that, if they lived longer, a number of children affected by trisomies 13 or 18 would develop germ-cell neoplasms. This might also be true for triploid fetuses who display PLAP immunopositivity in intratubular spermatogonia.

Most testicular growths are associated with, or begin as, intratubular CIS. This entity is clearly characterized by PLAP immunopositivity of all intratubular neoplastic germ cells in almost 100% of cases. Surprisingly enough, CIS is highly PLAP-positive while the adjacent invasive, neoplasm may not be so in every instance. Seminoma is 95% positive for all tumour cells with the possible exception of spermatocytic seminoma (Lange et al. 1982). We have demonstrated that for CIS and seminomas, the polyclonal antibody and both monoclonals tested worked equally well and that their immunopositivity was similarly located for a given tumour. We could not thus separate true PLAP from PLAP-like (germ-cell alkaline phosphatase) in our tumour material. Further work is in progress to characterize the ultrastructural localization of the marker. Interestingly enough, PLAP positivity of tumour cells decreased with increasing degree of differentiation. Teratomas were absolutely negative, while embryonal carcinomas and choriocarcinomas showed some immunostaining. However, immunopositivity was always restricted to selected tumour cells; these were syncytiotrophoblasts for choriocarcinomas, resembling thus the normal mature placenta. In embryonal carcinomas, the number of positive cells was usually small and while not unlike neoplastic isolated germ cells, others appeared as tumour cells within papillary fronds or labyrinthine cords.

A final comment must be expressed as regards ovarian germ-cell neoplasms, which are much less frequent than their testicular counterparts. However, it is noteworthy that Nouwen et al. (1987) included one dysgerminoma in their series: it was markedly PLAP-positive contrasting with the finding of Davies et al. (1985) and McDicken et al. (1985) who described ovarian teratomas (benign and malignant) which did not demonstrate PLAP immunopositivity.

We do not know the precise significance of PLAP or PLAP-like immunopositivity in tumour germ cells. It might be a re-expression (through gene derepression) of an embryonic property but this is not a factor which controls proliferation.

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